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Evaluation of *Babesia* Spp. Infection among Native Horses in Quezon Province, Philippines, Using Molecular and Microscopical Examination

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Abstract

The possible existence of equine piroplasmosis was investigated in the province of Quezon, Philippines. Blood samples of 269 horses from selected municipalities of Quezon Province were gathered. Parasitological test namely blood parasite examination was employed and the test yielded negative results for the presence of the parasite. Similarly, polymerase chain reaction assay was performed using *Babesia* genus-specific primers. PCR assay also resulted to all samples being negative for the disease.

The negative results are suggestive of a variety of causes specifically; low parasite concentration during sample collection, localization of the parasite in the endothelium, latent stage of infection, acquired immunity, drug intervention and true negative or complete absence of parasite in the blood. The abovementioned reasons are either characterized by very low parasitemia, no parasitemia or eventually complete disappearance of the parasites in the blood, all of which subsequently confounds the observance of the parasite.

Keywords: PCR, BPE, *Babesia*, Equine, Philippines

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Introduction

Horses used for the purposes of draft are part of a country's agricultural machinery. The National Statistics Office of the Philippines reported the equine population at an estimated 328,900 heads (NSO, 2002). Ticks, which may be carriers of the equine piroplasmosis-causing protozoa are known to infest these animals (Mandac, 1965). Furthermore, these tick vectors are distributed worldwide.

Equine piroplasmosis (EP), also known as biliary fever or gall sickness (Ikadai et al., 2001), is a vector-borne disease affecting horses, mules, donkeys and other equids. It is caused by intraerythrocytic protozoan parasites *Babesia caballi* and *Babesia (Theileria) equi*. Concurrent infections of both parasites are known to occur, however, *B. equi* infection is more commonly diagnosed (Soulsby, 1982). The disease is characterized by anemia, pyrexia, edema, icterus and hemoglobinuria leading to eventual loss of condition of the animal. Recovery is possible but recovered animals may become life-long carriers.

At present, several techniques have been employed in its diagnosis including blood parasite examination (BPE), serological tests (Hirata et al., 2004) and molecular methods such as polymerase chain reaction (PCR) assay (Sahagun-Ruiz et al., 1997).

In this study, the occurrence of equine piroplasmosis in horses from selected municipalities of Quezon Province was inspected using BPE and PCR assay. To the authors' knowledge, there are no reports of molecular studies on equine piroplasmosis in Quezon Province, Philippines. The information gained from this study will be useful in the better understanding of the distribution of the disease in the country.

Materials and methods

Horses

Municipalities from Quezon Province were randomly selected. Information regarding the horses' population from each chosen municipality was obtained from the Provincial Veterinarian's Office. Blood collection commenced with prior permission from the Provincial Veterinarian, Municipal Agricultural Officers, owners and initial approval of the Institutional Animal Care and Use Committee (IACUC) (Protocol No. 2008-037).

Sample size determination

Multi-stage sampling was implemented wherein municipalities from Quezon Province were primarily selected at random. The number of samples collected per municipality was determined following the work of Herd (1991) (Table 1).

Blood sample collection and examination

History of the animals was determined using a questionnaire form, prior to blood collection. Gauge-18 or 19 needles were used to draw 3-5ml of blood from jugular vein of the animal. Collected blood samples were placed in ethylenediaminotetraacetic acid (EDTA)-containing vacutainer tubes.

The blood smears were fixed in absolute methanol for 5 to 10 min and allowed to air-dry. Air-dried smears were immersed in diluted Giemsa-stain® for 30 min. Stained slides were washed with distilled water and air-dried (Coles, 1986). Slides were viewed under the 100x oil-immersion objective following the method of Pritchard and Kruse (1982). Parasites were identified as previously illustrated (Bork et al., 2003).

DNA extraction

DNA was extracted using the method as previously described (Sambrook and Russell, 2001, Baticados et al., 2005 and Baticados, 2010) with slight modifications. One to two hundred microliters (µl) of blood were mixed with the DNA extraction buffer containing proteinase K (100 µg/mL) in 1.5 ml microcentrifuge tube. Samples were then incubated at 55ºC for 12 h. Five hundred microliters phenol-chloroform-isoamyl alcohol (PCI) were added and mixed for 10 s at 2500 rpm using vortex mixer. Centrifugation at

14000 rpm for 5 min followed. The aqueous layer was transferred to a new microcentrifuge tube. Chloroform, in amounts equal to the sample, was added. The samples were mixed and centrifuged as previously described. The aqueous layer was transferred to a new tube with 10µl sodium acetate (NaCOOH) and crude DNA precipitation was performed by adding 1 ml 99.5% ethanol. Tubes were mixed vigorously and incubated in freezing temperature for 1 h. The samples were subjected to centrifugation for 10 min and afterwards, the fluid decanted. One milliliter of 70% ethanol was mixed with each sample and again followed by centrifugation at 14000 rpm for 10 min. The fluid contents of the tubes were decanted and excess fluid pipetted off. Tubes were air-dried and DNA resuspended by addition of 50 µl Tris-EDTA (TE) buffer. Samples were stored at -40ºC until needed for molecular processing.

PCR assay

Polymerase chain reaction amplification was performed with 25 µL reaction mixture containing 1 µL of each DNA template, 200 µM of dinucleoside triphosphates (dNTP) mixture (Roche, USA), 1 U of *Thermus aquaticus* (Taq) DNA polymerase, 10× PCR reaction buffer (Roche, USA) and 20 pmoles each of *Babesia* genus-specific forward and reverse primers (Eurogentec AIT, Singapore) (Figure 1A). Autoclaved distilled water was used as negative control. PCR assay tubes were ran in a thermal cycler (Touchgene Gradient, Techne Cambridge, UK) programmed at 40 cycles with initial denaturation of 95ºC for 5 min, denaturation at 95ºC for 1 min, annealing at 45ºC for 1 min, extension at 72ºC for 1 min, final extension at 72ºC for 5 min and held at 4ºC for 10 min.

Gel electrophoresis

Polymerase chain reaction products together with 50 base pair (bp) DNA ladder (New England Bio-Labs Inc., USA) and negative control (distilled water), were resolved in in 1.8% Tris-acetate-EDTA (TAE) agarose gels submerged in TAE buffer. Gels were visualized by addition of ethidium bromide stain and viewed using Digidoc-IT Imaging System (UVP California, USA).

Results and Discussion

Horses

Native draft horses from 11 municipalities (San Antonio, Tiaong, Dolores, Lucban, Sampaloc, Pagbilao, Atimonan, Plaridel, Gumaca, Alabat and Perez) of Quezon Province were used in the study (Figure 1B). A total of 269 horses were sampled of which 151 were male and 118 were female. According to owners, the male horses were preferred over female horses since males could carry heavier loads for longer working hours and were not liable to be inoperative due to reasons such as pregnancy. The horses from the province of Quezon were mainly used for draft purposes such as hauling produce like coconuts, coconut lumber and water. On the average horses were aged six years old, the youngest being eight months old and the oldest at 20 years old. In addition, 134 horses were aged between 5-10 years, an age probably considered most optimal for work by owners (Table 1).

Blood parasite examination

In this study, BPE was used to detect the presence of the parasites causative of equine piroplasmosis. Blood parasite examination resulted to none of the slides showing *Babesia* spp. tropozoite or merozoite forms in all blood films (Table 1).

Although demonstration of the parasites in blood film examination is the best and most sustainable method for on-site diagnosis (Hong et al., 1997) and is definitive; it is only possible during the stage of high parasitemia and is insensitive (Hirata et al., 2004, Asgarali et al., 2007). Parasitemia is evident in acute cases particularly during febrile periods but not in convalescing or carrier animals (Jain, 1986). Upon onset of the latent stage, there may be complete disappearance of the parasites in the blood (Holman et al., 1993).

BPE can detect the agent of equine piroplasmosis at parasitemia of 0.001% (Posnett et al., 1991). BPE is useful, but parasitemia is fleeting and negative results can be misleading (Mansmann et al., 1982).

Table 1: Frequency Distribution of Signalment, BPE and PCR Results of 269 Native Horses from Different Municipalities of Quezon Province, Philippines.

Municipality	(N)	(n)	Sex		Age				BPE (+)	PCR (+)
			B	≅	<5 yrs	5-10 yrs	11-15 yrs	15-20 yrs		
Gumaca	735	63	33	26	22	30	4	3	0	0
Dolores	683	55	13	36	24	21	3	1	0	0
Tiaong	296	24	7	17	19	5	0	0	0	0
Sampaloc	227	23	22	0	2	19	1	0	0	0
Lucban	366	31	24	7	13	14	3	1	0	0
Atimonan	661	55	14	8	7	12	2	1	0	0
Plaridel	122	14	7	7	9	4	1	0	0	0
Pagbilao	323	30	21	7	10	18	0	0	0	0
Alabat	24	6	4	2	1	4	1	0	0	0
Perez	41	7	5	1	4	2	0	0	0	0
San Antonio	260	24	1	7	1	5	2	0	0	0
TOTAL	**3738**	**332**	**151**	**118**	**112**	**134**	**17**	**6**	**0**	**0**

Polymerase chain reaction assay

PCR assay represents a new breed of technological application to animal disease detection. Amplification of certain sequences of the pathogens nucleic acids are used for the purpose of diagnosis (Viljoen et al., 2005).

Of the 269 samples ran in PCR assay, none were found positive for equine piroplasmosis (Table 1). Specifically, the 650 base pair band, expected for the genus *Babesia* (Inokuma et al., 2003), were not observed after gel electrophoresis and photo documentation under ultraviolet (UV)-light. Moreover, the assay was performed for four times but yielded the same results.

PCR assays by Xuan and co-workers (1998) detected *B. caballi* at parasitemia of 0.000001%. Bashiruddin and co-workers (1999) detected *B. equi* at parasitemia of 0.000083% and Al Hassan and co-workers (2007) detected *B. equi* at parasitemias of 0.0001%.

Based on the outcome, there are several possibilities that caused none demonstration of the parasite: First, possible latent stage of the infection wherein parasites are minimal and undetectable; second, acquired immunity of previously infected horses; third, very minute quantities of parasite DNA could have led to problems with PCR amplification of the organism (Viljoen et al., 2005); fourth, piroplasms could have localized in the capillary endothelium where the parasites also have an inherent affinity, thus, were absent in the peripheral circulation (Brown and Bertone, 2002); fifth, it is a common phenomenon that false negatives can still occur in PCR assay (Bose et al., 1995); sixth, true negative or complete absence of the parasite and lastly, drug intervention that may contribute to the early termination of subclinical infection and further lead to immunity to reinfection for a certain period of time (Soulsby, 1982).

In connection, it was noted that a number of horses in Quezon province were previously treated with drugs against surra, quinapyramine (Triquin®).

Diagnoses of equine piroplasmosis by both BPE and PCR have significant consequences in field and laboratory settings but the difference in sensitivity is considerable.

Although BPE is simpler and readily available in field and lab conditions, its sensitivity is a fraction of that of PCR assay,

A

Primer	Sequence (5'-3')	Gene target	Expected band size	Reference
Babesia-F	GTGAAACTGCGAATGGCTCA	28S	650 bp	Inokuma
Babesia-R	CCATGCTGAAGTATTCAAGAC	rRNA		*et al.*, 2003

B

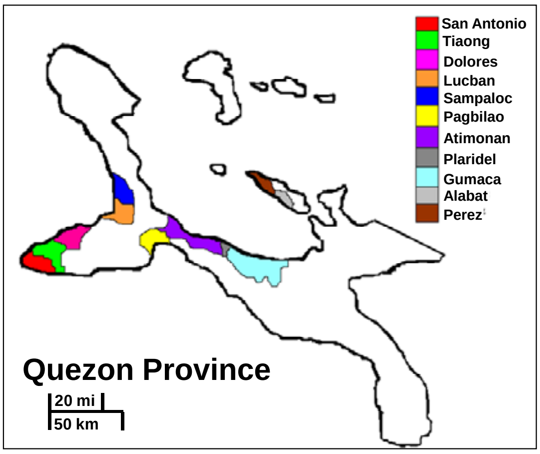


Figure 1: *Babesia* genus-specific primers and expected amplicon size (A). Map of Quezon Province and selected municipalities (B).

which is 100 times more sensitive (Bose et al., 1995). Using BPE alone, positive samples can be identified only 1.5% of the time (Kumar et al., 2008). On the other hand, while PCR assay is more sensitive and specific primers can properly identify the parasites, PCR assay is complex requiring appropriate equipment, training and personnel.

Furthermore, BPE has long been used for the purpose of animal disease diagnosis and has been proven useful yet, at times, insufficient. PCR assay, on the other hand, is a relatively new technique which is still further being developed and updated.

It should be noted that ticks of the genera *Rhipicephalus* and *Boophilus* are implicated in equine piroplasmosis transmission in other nations and potential vectors belonging to these genera are present in the country. This includes *R. sanguineus* (Mandac, 1965 and Ramirez, 1986), *R. haemophysaloides* (Pippin, 1966 and Walker et al., 2000) and *B. microplus* (Mandac, 1965 and Mehlhorn and Schein, 1998) whose ability to transmit the disease under Philippine conditions is yet to be confirmed.

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بررسی آلودگی با گونه های بابزیا در اسب های بومی استان کوزون فیلیپین از طریق روشهای مولکولی و میکروسکوپی

فلچر پی دل وال، آبیگیل ام باتیکادوس و وارن ان باتیکادوس

گروه علوم پاراکلینیکی دامپزشکی ، دانشکده دامپزشکی دانشگاه فیلیپینز لوس بانوس، لاگونا، فیلیپین

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چکیده

در این مطالعه، حضور احتمالی پیروپلاسموز اسبی در استان کوزون فیلیپین مورد بررسی قرار گرفت. بررسی هـای انگـل شناسـی بـر از طریق آزمایش انگل شناسی خون بر روی ۲۶۹ راس از دامهای مناطق مختلف این استان صورت گرفت. همچنین تست PCR با اسـتفاده از پرایمر های مخصوص جنس بابزیا بر روی این نمونه ها انجام گرفت که نتایج منفی تمامی آزمایشات حاکی از عدم حـضور بابزیـا در نمونـه های مورد بررسی بود. این نتایج منفی می تواند ناشی از فاکتور های مختلفی باشد، از جمله: تعداد پایین انگل در حین زمـان نمونـه گیـری، تجمع انگل ها در اپیتلیوم، مراحل پیشرفته بیماری، ایمنی اکتسابی، تداخلات دارویی و نیز منفی واقعی یا به عبارت دیگر عدم وجود آلودگی. دلایل ذکر شده هم می توانند باعث کاهش تیتر انگل در خون شوند و هم می توانند سبب عدم حضور انگـل در خـون و بطـور کلـی عـدم رویت آن گردند که می توانند از عوامل مهم مخدوشگر در تشخیص این بیماری باشند.

واژگان کلیدی : PCR، BPE، بابزیا، اسب، فیلیپین